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The Wnt family of secreted signaling molecules plays crucial roles in mammary tumorigenesis and embryonic development. The *frizzled* family of serpentine receptors has been shown to be capable of transducing the Wnt signal suggesting that a *frizzled* protein may encode for the receptor for Wnt-1.

This hypothesis was tested using the *Xenopus* axis duplication system using a co-injection strategy with pools of *fz* molecules and a Wnt1-CD8 molecule. The ability of the *fz* molecules in one of the pools to mediate the secondary axis formation of Wnt1-CD8 has lead to the identification of two *fz* proteins which may potentially serve as the Wnt-1 receptor. However this strategy has been failed to delineate a single *frizzled* protein as a receptor suggesting there may be redundant functions of various *frizzled* molecules to transduce this signal. Experiments are now in progress to attempt to identify molecules that interact with the cytoplasmic portion of the *frizzled* receptor and *dishevelled*, a downstream effector, in an effort to understand the transduction of the Wnt signaling. The elucidation of downstream effectors for the Wnt-1 signaling molecule can provide valuable insights into the molecular nature of mammary tumor formation and embryonic development.

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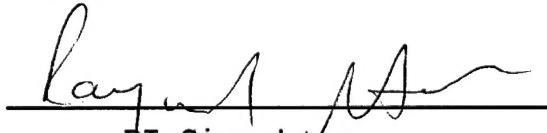
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## Introduction

The Wnt gene family encodes secreted signaling molecules that play important roles in mammary tumorigenesis and embryonic development. Delineation of the mechanisms of Wnt-1 signaling can provide insights into the molecular nature of mammary tumor development and early embryonic pattern formation. Our current understanding of the Wnt signal transduction cascade proposes that a member of the *frizzled* proteins may function as the receptor of the Wnt-1 ligands. I have been screening pools containing identified *frizzled* molecules using the established *Xenopus* axis duplication system to determine if such a *frizzled* molecule can be identified as the receptor for Wnt-1. Furthermore I have been screening for interacting molecules for the *frizzled* and *dishevelled* proteins using the yeast two- hybrid system to try and elucidate the biochemical basis of the Wnt signal transduction cascade.

The identification and elucidation of the receptor for the Wnt-1 signaling molecule and downstream components of its signal transduction machinery can provide valuable insights into how this ligand functions in mammary tumor formation.

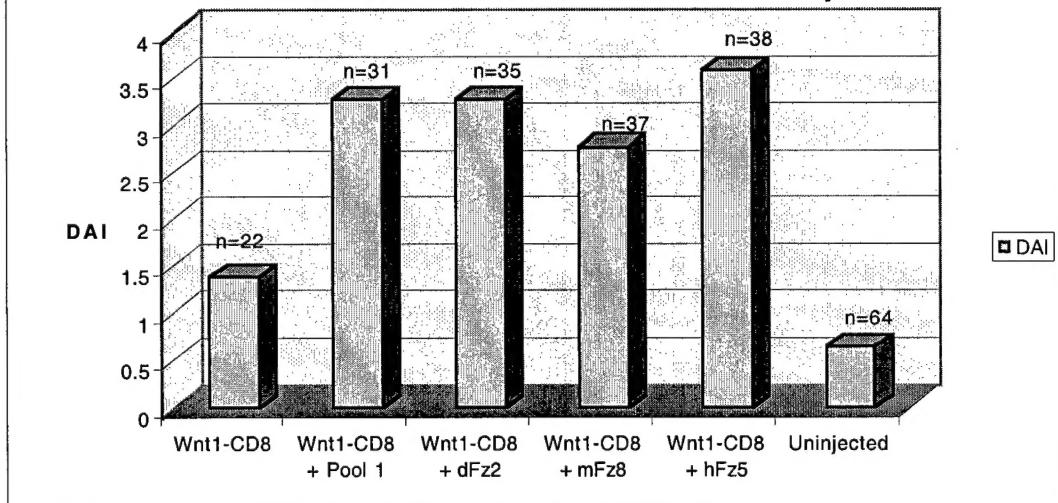
## BODY

### Major Project covering Task 1 in Statement of Work (Months 1-24)

I have used the *Xenopus* secondary axis duplication assay to screen for *frizzled* molecules that may function as the receptor for Wnt-1. As reported in my last renewal, I was able to identify from pools of *frizzled* molecules, two *frizzled* proteins that can in the presence of suboptimal concentration of Wnt-CD8 transduce this signal, see figure 1 (figure 5 of my last report):

**Figure 1**

**Effects Of Pool 1 On UV Ventralized Embryos**



I have repeated these experiments and have obtained similar results with both dFz2 and mFz8 being capable of transducing the Wnt signal both in secondary axis duplication assays and also rescue of UV ventralized embryos.

These data demonstrate a technical problem which I did not expect to encounter with the original proposal, that is the *Xenopus* secondary axis duplication assay may not be able to resolve the finer aspects of interaction of the Wnt-1 protein with a specific *frizzled* protein. This system is powerful in demonstrating that Wnt-1 can synergize with *frizzled* proteins but as I found two independent *frizzled* proteins can synergize I am unable to distinguish which one of the proteins may be the true receptor for this ligand. Perhaps the combination of biochemical binding assays with the results from the secondary axis duplication assay would be able to demonstrate the nature of the interaction based on binding affinity. But these experiments cannot be attempted because of the inability of obtaining soluble Wnt-1 protein. Furthermore it is equally likely that both of these *frizzled* proteins I have identified in the secondary axis duplication assay will bind to Wnt-1 with varying affinity and the argument that the *frizzled* protein which binds with the stronger affinity is the true receptor may not be a conclusive argument.

My data therefore demonstrates that two *fz* proteins may mediate the secondary axis duplication in the presence of Wnt-1 and this suggests that more than one *fz* protein may serve as a receptor for this molecule. This finding may be a direct reflection of the recent finding that the Frizzled and dFz2 proteins may serve redundant functions in mediating the effects of *wg* (Bhat, 1999 and Kennerdell and Carthew, 1998).

### **Minor project (1) not outlined in Statement of Work**

A minor project that I have been addressing is directly related to the Wnt-1 signaling but not written in my submitted Statement of Work. This project involves the attempts to identify molecule(s) which interact with the carboxyl terminus of the hFz5 receptor which has been shown to induce secondary axis formation in the presence of Wnt5a (He et al., 1997). The hypothesis proposed is that as activation of the hFz5 receptor can mimic the axis induction effects seen with Wnt-1, it may directly bind to substrates involved in mediating the Wnt-1 signal transduction cascade. Removal of this cytoplasmic portion of the receptor was unable to mediate secondary axis duplication in the presence of Wnt5a suggesting that this portion of the receptor was required for transducing the Wnt signal (data not shown).

I have performed a yeast two-hybrid screen using the hFz5 carboxyl terminus as the bait and fused with the Gal-4 DNA binding domain, this sequence encompasses the terminal 70 amino acids of the hFz5 protein. For the construction of the bait, the last four

amino acids (LSHV) of the carboxyl terminus was removed. These last four amino acids resemble the classical recognition site for binding of PDZ containing proteins thought to be involved in clustering or anchoring proteins on the plasma membrane. The removal of this sequence should therefore provide a screen to remove any putative positive clones only involved in clustering the receptor. The removal of these terminal four amino acids was found not to interfere with the receptors ability to signal to downstream effectors in *Xenopus* secondary axis duplication in synergy with Wnt-5a (data not shown).

A library containing cDNAs from rat brain library, from Clonetech, was used for the screen and 3.0 million independent clones were screened. From this screen, 42 positives were obtained which conferred His prototrophy and were Lac-Z positive. These positives clones were systematically rescued from the yeast and sequenced. The identities of these clones are summarized in Table 1:

**Table 1**

| <b>Clone # Identity</b>                    | <b>Clone # Identity</b>              |
|--|--------------------------------------|
| 1 cytochrome oxidase                       | 18 collagen alpha-2 (XI) chain       |
| 2 cytochrome oxidase                       | 19 ribosomal protein L30             |
| 3 aromatase                                | 20 28S ribosomal RNA                 |
| 4 transferrin                              | 21 28S ribosomal RNA                 |
| 5 CDC8 (5' non coding)                     | 22 Rat proteasome RN3                |
| 6 Rat protein kinase C-binding protein Nel | 23 cytochrome oxidase                |
| 7 ribosomal protein L30                    | 24 cytochrome oxidase                |
| 8 aspartate aminotransferase               | 25 NADH:ubiquinone oxidoreductase    |
| 9 ribosomal protein L30                    | 26 RFX1 dna binding protein          |
| 10 cytochrome oxidase                      | 27 cytochrome oxidase subunit I      |
| 11 cytochrome oxidase                      | 28 16S RNA                           |
| 12 Rat rS-Rex-s mRNA                       | 29 ribosomal protein L30             |
| 13 Rat proteasome RN3                      | 30 collagen alpha-2 (XI) chain       |
| 14 microtubule-associated protein 1A       | 31 cytochrome oxidase                |
| 15 28S ribosomal RNA                       | 32 microtubule-associated protein 1A |
| 16 28S ribosomal RNA                       | 33 cytochrome oxidase                |
| 17 Rat L1 retrotransposon                  | 34 ribosomal protein L30             |

The positives obtained from this screen revealed that no clone of interest was obtained, in fact all of the clones were false positives as they were later demonstrated to be trans-activators of both the His and Lac-Z gene. These results suggested that either the bait was not folding correctly to adapt a correct conformation for interaction or this fragment was insufficient for interaction with transducing molecules. It is plausible that cytoplasmic loops in conjunction with the carboxyl cytoplasmic tail are together required for the binding of effector molecules. However this hypothesis cannot be tested as there is no way of making such a bait for the two-hybrid system.

This screen therefore was unable to elucidate candidate effector molecules capable of transducing the Wnt-1 signal.

### **Minor project (2) not outlined in Statement of Work**

A second minor project that I have been addressing also involves the attempt to find molecules that may transduce the Wnt signal downstream of the *frizzled* receptor. This project involves the attempts to identify molecule(s) which interact with the dishevelled protein, a downstream molecule genetically and biochemically linked to the signal cascade. The rationale behind this screen was to potentially find a molecule that binds to dishevelled that could mediate the Wnt signal cascade allowing me to work back up to the receptor level. To accomplish this screen, two independent fragments of the dishevelled protein, mouse dishevelled 2, were used as baits, the DIX domain and the PDZ domain. The same rat brain cDNA library as for the hFz5 receptor screen was employed.

For the DIX domain screen, 2.15 million independent clones were screened and 125 positives were obtained, rescued from the yeast and sequenced. These positives are summarized in table 2

**Table 2**

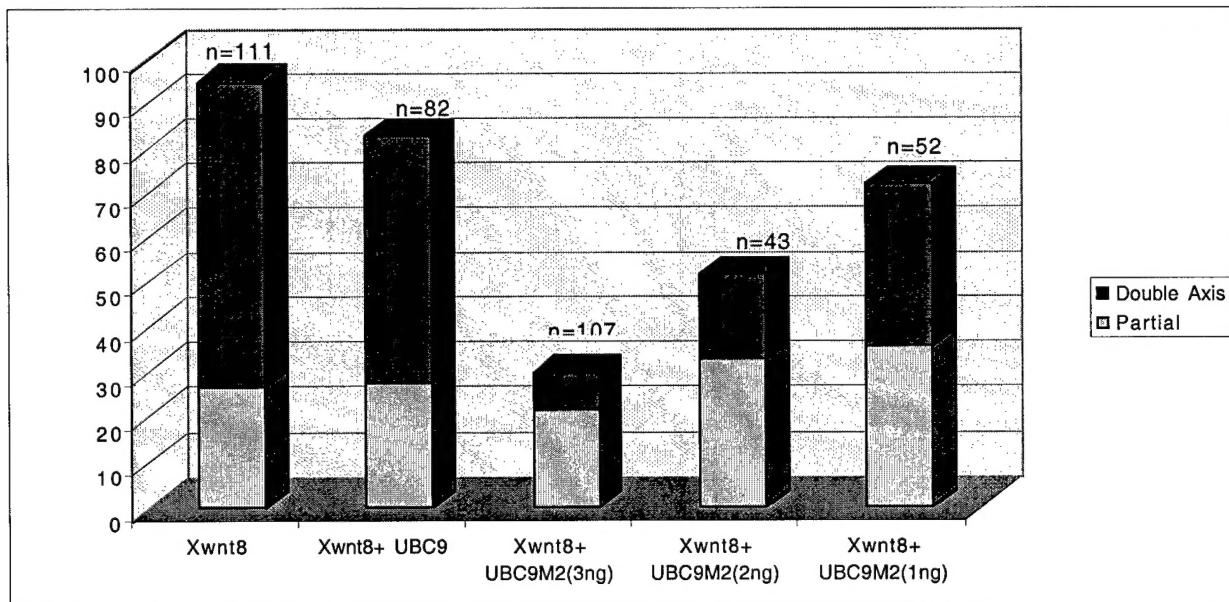
| <b>#of clones</b> | <b>Identity</b>                     |
|-------------------|-------------------------------------|
| 100               | Ubiquitin conjugating enzyme 9      |
| 3                 | rat dishevelled 3                   |
| 3                 | protein arginine methyl transferase |
| 1                 | ribosomal protein L23               |
| 2                 | mouse epitope                       |
| 2                 | elongation factor e2F               |
| 7                 | GSK-3 binding protein               |
| 1                 | $\beta$ -globin                     |
| 1                 | transretyrin                        |
| 5                 | could not rescue                    |

The finding that the dishevelled proteins can homodimerize was published at the time that this screen was ongoing, see Kishida et al. 1999, so I concentrated on the UBC9 clone. Overexpression of this clone ventrally was unable to induce a secondary axis when injected at doses ranging from 1-3 ng per embryo. Expression of a mutant version, Banerjee et al., 1995, was unable to specifically block formation of endogenous axis when expressed dorsally. Furthermore the UBC9 or mutant clone was unable to block Wnt mediated secondary axis formation specifically. As figure 2 shows, UBC9M2 was able to cause some interference with secondary axis formation in the presence of 10pg of Xwnt8 but at very high concentrations. To verify whether this effect was real, I turned to animal cap experiments, which provide a much more sensitive assay for the ability of UBC9, or UBC9M2 to induce or block genes known to be induced by the active Wnt

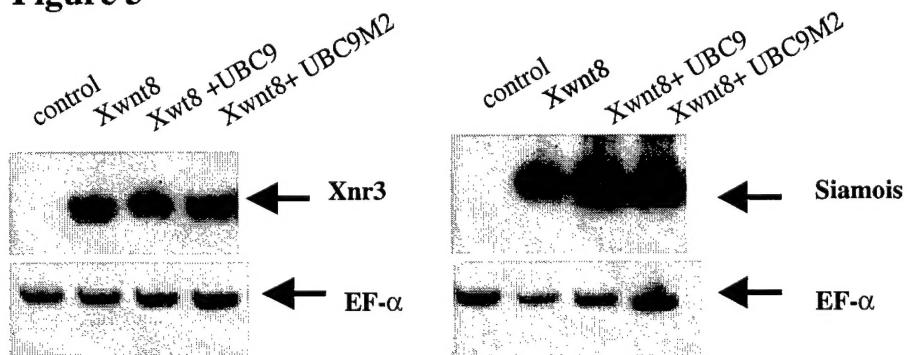
cascade. The results of this assay demonstrate that UBC9 or UBC9M2 did not interfere with Xwnt-8's ability to induce these genes, see figure 3.

**Figure 2**

**Effects of UBC9 and UBC9M2 on Wnt8 signaling in Secondary axis duplication assay**



**Figure 3**



These results therefore suggest that UBC9 though is a true interacting dishevelled protein may not have an essential role in the Wnt signal transduction cascade.

For the PDZ domain screen, 3.8 million independent clones were screened and 54 positives were obtained, rescued from the yeast and sequenced. These positives are summarized in table 3.

**Table 3**

| #of clones | Identity                 |
|------------|--------------------------|
| 1          | 8 novel gene called HeXi |
| 2          | novel gene called Natas  |
| 13         | cytochrome oxidase       |
| 1          | nuclear cyclophilin      |
| 2          | DNA repair enzyme        |
| 2          | KU autoantigen           |
| 1          | filamin A                |
| 3          | dynein                   |
| 1          | HSP40                    |
| 11         | could not rescue         |

From this screen, two interesting clones were obtained, HeXi and Natas. I am now examining the roles of these two clones on the Wnt signal transduction pathway by first obtaining the full length clones, looking at tissue distribution by Northern Blot analysis and examining the ability of these clones to either activate secondary axis formation or their ability to block Wnt mediated secondary axis formation.

### **Summary**

The experiments performed thus far encompassed the project outlined in the Statement of Work for months 1-24. The data presented summarizes the continued inability of the proposed experimental approach to identify a single receptor for the Wnt-1 molecule. The data from these experiments however do strongly suggest that Wnt-1 may be able to mediate its signaling abilities by interacting with more than one *frizzled* molecule. Concurrent with these finding are the published data from Drosophila demonstrating redundancy in the roles of the two *frizzled* molecules in mediating wingless signaling.

The use of the powerful genetic screen for the identification of interacting proteins, the yeast two-hybrid system, with the carboxyl portion of the *frizzled* receptor was unable to identify any such molecules. This finding strongly suggests that additional proteins of the cytoplasmic loops of the receptor may be required for the binding of the effector molecules for mediating signaling. Such a requirement has been demonstrated for G-protein coupled receptors of which the *frizzled* family closely resembles in its predicted tertiary structure.

In a continued effort to understand the signal transduction mediated by Wnt-1, the first downstream component, *dishevelled*, was separated into two fragments and used as baits for a two hybrid screen. The N-terminal bait was unsuccessful in identifying new components but verified that this protein functions as a homodimer in identifying *dishevelled* as an interacting molecule. The PDZ domain proved to be the best fragment to use as a bait as two novel proteins were identified as interacting molecules. I am now concentrating my efforts to understand the roles of these proteins in mediating this signaling cascade.

Continued efforts at understanding the mechanisms of Wnt-1 signal transduction can help to elucidate the complex role of this signaling molecule in tumorigenesis and embryonic development,

### **Key research accomplishments**

- identified two *frizzled* molecules that can transduce Wnt-1 signaling.
- demonstrated that additional regions apart from the C-terminal protein of hFz5 receptor may be required for receptor function.
- have identified two novel molecules that bind to the PDZ domain of the *dishevelled* protein that may be important for Wnt-1 signal transduction.

### **Reportable Outcomes**

- none

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